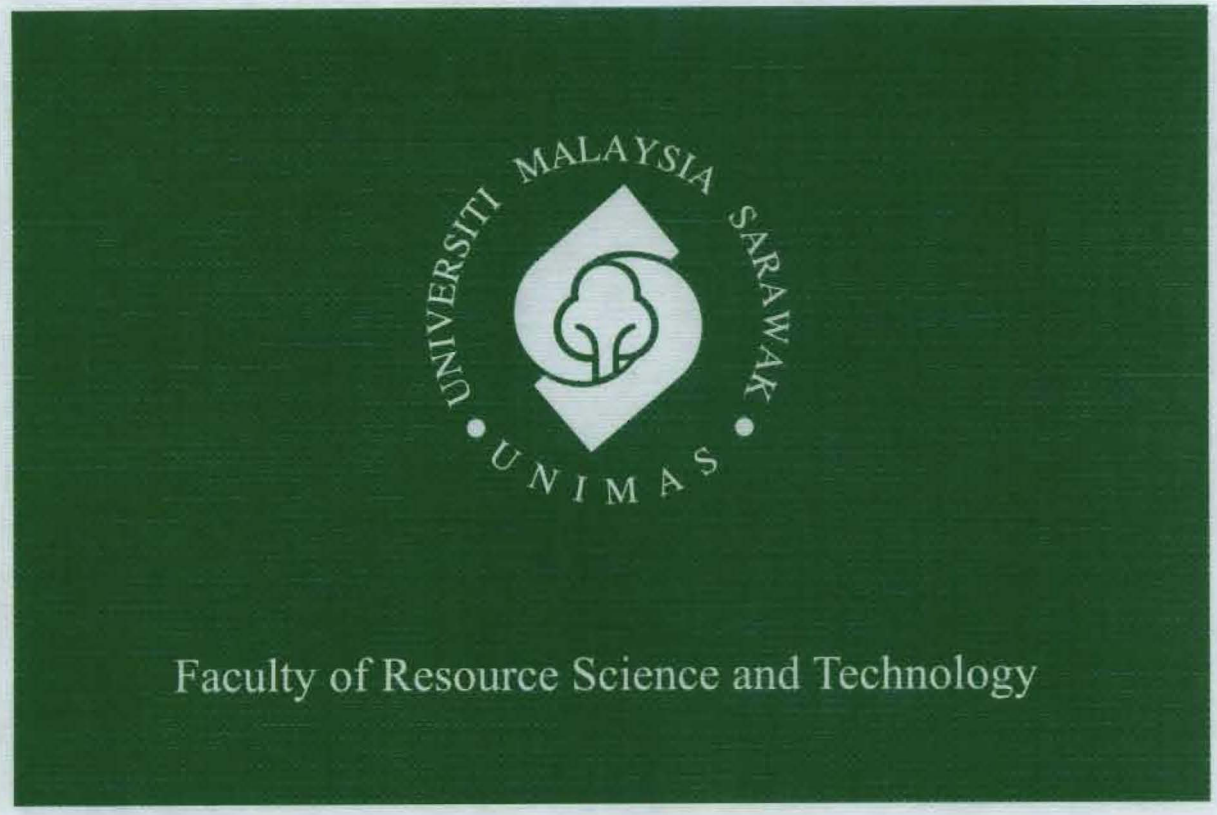

Heterologous Expression of Xylanase Gene from *Klebsiella pneumoniae* in *E. coli* BL21 (DE3)

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Bachelor of Science with Honours
(Resource Biotechnology)
2012

Heterologous Expression of Xylanase Gene from *Klebsiella pneumoniae* in *E. coli* BL21 (DE3)

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25176

A thesis submitted in partial fulfilment of the requirement for the degree of Bachelor of Science with Honour (Resource Biotechnology)

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6 July 2012

ACKNOWLEDGEMENT

First of all, I would like to thank my supervisor Dr. Mohd Hasnain Md Hussain for the opportunity given to me to do this project under him, as well as continuous guidance and support in helping me completing this project successfully. Apart from that, I would like to thank Dr. Hairul Azman Roslan and Dr. Awang Ahmad Sallehin Awang Husaini either for their guidance, contribution of ideas for this project or for allowing me to utilize their chemicals and reagents as well as their laboratory. I would also like to give my thanks to all the postgraduate students from the Proteomics Lab, that is Siti Izyan Liyana Binti Kamarul, Yan Wei Jie, and Nikson Chong Fatt Ming especially for spending their precious time and giving valuable help and support in the completion of this project. Besides that, I would like to give my thanks to all the postgraduate students from the other labs, especially the Genetic Engineering Lab under Dr. Hairul Azman Roslan and Molecular Genetics Lab under Dr. Awang Ahmad Sallehin Awang Husaini for the continuous and immediate help every time it was needed. Apart from that, I would like to thank all my lab mates and friends for the understanding, motivation and co-operation given in the completion of this project. My precious thanks also for my mother, Sharifah Binti Hamid and my father, Zainol Bin Zain, as well as my sister, Shazlin Binti Zainol for the believe they had in me and continuous support and motivation, which give me the strength to complete this project. Lastly, I would like to thank everybody who has helped in this project.

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LIST OF ABBREVIATIONS

AGE	agarose gel electrophoresis
AP	alkaline phosphate
BCA	bicinchoninic acid
BCIP	(5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate
dNTP	deoxynucleoside triphosphates
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	luria bertani
MES	2-(N-morpholino)ethanesulfonic acid
NBT	nitro blue tetrazolium
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	super optimal broth with catabolite repression
TCP	total cell protein

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Heterologous Expression of Xylanase Gene from *Klebsiella pneumoniae* in *E. coli* BL21 (DE3)

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ABSTRACT

A xylanase DNA sequence with a total length of 642 bp was isolated from a xylanolytic *Klebsiella pneumoniae* bacterium in order to attempt heterologous expression in *E. coli* BL21 (DE3). A xylanase gene primer was designed with the addition of *Bam*H1 and *Eco*R1 restriction enzyme sites in order get a full xylanase gene that is in-frame with pSTAG expression vector. The isolated xylanase was amplified using the designed xylanase primer through PCR, then cloned and expressed in *E. coli* BL21 (DE3). *In-silico* characterization showed that the recombinant xylanase without S-tag has a molecular weight of 23.9 kDa and a pI of 9.32. The signal peptide cleavage site for the recombinant xylanase and its native xylanase was predicted to be between residues 61 and 62, and residues 28 and 29 respectively. The activity of crude recombinant xylanase from freeze and thaw method and sonication method is 2.015 U/mL and 0.904 U/mL respectively, which is higher than the crude native xylanase activity showing only 0.642 U/mL at maximum. Staining of the birchwood xylan agar plate with Congo red showed clearing zone around *E. coli* BL21 (DE3) that has positive recombinant xylanase even without the addition of IPTG, implying leaky expression had occur, and that *E. coli* BL21 (DE3) secretion system had recognizes the signal sequence of the native xylanase and proceeds to cleave it, then secreting out the mature protein into culture medium.

Key Words: *Klebsiella pneumoniae*, heterologous expression, cloning, recombinant xylanase

ABSTRAK

Jujukan DNA xylanase dengan panjang keseluruhan 642 bp telah diasingkan daripada bakteria pengurai xylan Klebsiella pneumoniae untuk cubaan menghasilkan xylanase daripada E. coli BL21 (DE3). Primer gen xylanase telah direka dengan tambahan jujukan enzim BamH1 dan EcoR1 bagi mendapatkan gen xylanase yang penuh dalam rangka bacaan pSTAG vektor. PCR telah dijalankan menggunakan jujukan DNA xylanase dan primer gen xylanase yang telah direka. Hasil PCR kemudian melalui proses pengklonan ke dalam E. coli BL21 (DE3) dimana xylanase rekombinan kemudian dihasilkan. Pencirian secara in-silico menunjukkan bahawa xylanase rekombinan tanpa S-tag mempunyai berat molekul sebanyak 23.9 kDa dan ramalan titik isoelektrik (pI) 9.32. Isyarat peptida untuk xylanase rekombinan diramalkan antara asid amino 61 dan 62, dan asid amino 28 dan 29 untuk xylanase yang matang. Aktiviti xylanase rekombinan mentah daripada kaedah pembekuan dan pencairan adalah 2.015 U/mL, dan untuk kaedah sonikator adalah 0.904 U/mL. Kedua-duanya lebih tinggi daripada xylanase asli mentah yang menunjukkan hanya 0.642 U/mL aktiviti maksimum. Pewarnaan xylan birchwood agar dengan Congo merah menunjukkan zon kosong sekitar E. coli BL21 (DE3) yang mengandungi xylanase rekombinan yang positif walaupun tanpa kehadiran IPTG. Keadaan ini menunjukkan bahawa kemungkinan berlakunya penghasilan protein tanpa kawalan, dan sistem rembesan E. coli BL21 (DE3) mungkin mengecam isyarat peptida xylanase asli, kemudian memotong jujukan dan merembeskan keluar protein yang matang ke dalam medium kultur.

Kata Kunci: Klebsiella pneumoniae, penghasilan protein, pengklonan, xylanase rekombinan

1.0 INTRODUCTION

Malaysia generates tones of abundant agricultural waste annually, and some of these waste materials include oil palm trunks and fronds, coconut fibers and meal, cocoa pods, rubber wood dusts and many other (Pang *et al.*, 2006). Xylan, which consist of β-1,4-linked xylopyranosyl residues, is the second major abundant polysaccharide and an important component that made up the plant cell wall, as well as agricultural waste. Due to heterogeneity and complexity of xylan structure, the complete hydrolysis of xylan is carried out by xylanolytic enzymes system, which consists of endoxylanase, β-xylosidases, glucuronidases, etc. (Kuhad & Singh, 2007).

In this study, the focus was on endo-1,4-β-D-xylanase (EC 3.2.1.8). Xylanase cleave the xylan backbone at 1-4 carbon linkage to produce xylooligosaccharides and xylose (Kulkarni *et al.*, 1999). Various organisms have been reported to be able to produce xylanase such as bacteria, fungi, yeast,, protozoans, marine algae, snails, crustaceans, insects and seeds (Kuhad & Singh, 2007). Xylanase are mostly classified based on sequence similarities into two family of glycosyl hydrolase, which is family 10 and family 11 (Henrissat, 1991).

Xylanase attracted research interest because of its high potential in industrial applications. From biotechnology perspective, xylanase is an important enzyme since it can work alone or with the combination with others enzyme. In addition, xylanase thermal stability makes it suitable in a number of industrial applications (Te'o *et al.*, 2000). One of many useful applications of xylanase is in paper and pulp industry where it is use in kraft process and biobleaching (Kulkarni *et al.*, 1999; Helianti *et al.*, 2008).

Heterologous expression of gene encoding β-1,4-endoxylanase in *E. coli* BL21 (DE3) was performed in this study. The xylanase gene was isolated from xylanolytic *Klebsiella pneumoniae,* a bacterium that had been locally isolated from soil at the vicinity of the sago plantation by Hussain *et al.* (2011). So far, there are no research publications that described the isolation of xylanase gene from *K. pneumoniae*. Xylanase gene is commonly isolated from genus *Bacillus*, thus the isolation of xylanase gene from *K. pneumoniae* suggests that xylanase might be naturally occurring among soil-inhabiting microbes. The isolated xylanase gene is from family 11 glycoside hydrolase and showed similarity to *Bacillus* sp., although 16 rRNA showed that the bacterium closely resembled *Klebsiella*. *In-silico* characterization of the isolated xylanase gene showed that it has an open reading frame (ORF) of 642 bp encoding 213 amino acids, and are predicted to code for xylanase with a molecular weight 23.3 kDa with an isoelectric point of 9.44, which is quite similar to the xylanase isolated by Jalal *et al.* (2009) from *B. subtilis*, as well as Helianti *et al.* (2008) and Lee *et al.* (2008) from *B. licheniformis*.

Recombinant xylanase characteristics have been analyzed and compared to its native xylanase in quite a large number of research publications under the varying degree of temperature and pH. The crude xylanase enzyme activity for the isolated *K. pneumoniae* is relatively low, but the recombinant xylanase in this study is expected to show a high increase in activity once expressed and purified. The assumption is based on research done by Jalal *et al.* (2009), Helianti *et al.* (2008), and Lee *et al.* (2008), since their recombinant xylanase showed high increase in activity compared to the activity by native xylanase.

Recombinant xylanase had been reported to be distributed in extracellular, intracellular and periplasmic fractions in genus *Bacillus* (Huang *et al.*, 2006). Apart from that, recombinant xylanase were commonly found to be in insoluble fraction of

cytoplasmic, hinting that even after optimization the expression of xylanase gene produces protein that is insoluble and accumulates in inclusion bodies (Yin *et al.,* 2008). Generally, the production of xylanase through recombinant technology is for economical benefits aiming toward higher productivity and trouble-free downstream processing in fermentation (Farliahati *et al.*, 2009). Besides that, recombinant xylanase can be used for cellulose-free xylanase production in case of maintaining the cellulose structure as well as providing tolerances to various cations (Lee *et al.,* 2008)

Previous attempt to express locally isolated gene had been carried out by Rajamanikam (2010), but failed due to improper framing (frameshift) of cloned xylanase gene to *LacZ* promoter since he did not have the full sequence of recombinant pET41(a)-Xyn. Thus, a hypothesis was made in which *E. coli* BL21 (DE3) can express xylanase gene once the gene has been inserted in-frame inside the pSTAG expression vector. After that, the characteristic of the recombinant xylanase protein expressed could be determined.

Hence, a study regarding heterologous expression of xylanase gene from *K. pneumoniae* in *E. coli* BL21 (DE3) were conducted with the objective to express the isolated xylanase from *K. pneumoniae* in *E. coli* BL21 (DE3) and to determine the characteristic of the recombinant xylanase protein expressed. To accomplish this, a xylanase gene primer containing additional *Bam*H1 and *Eco*R1 restriction enzyme sites was designed to get a full xylanase gene that is in-frame with pSTAG expression vector. The isolated xylanase was amplified using the designed xylanase primer, cloned and expressed in *E. coli* BL21 (DE3) accordingly. *In-silico* characterization was carried out for the recombinant xylanase sequence. Qualitative assay using Congo red agar staining were performed to check for xylanolatic activity. Determination of the crude recombinant xylanase activity was also conducted in this study.

2.0 LITERATURE REVIEW

2.1 Xylan

Xylan, which consist of β-1,4-linked xylopyranosyl residues, is the second major abundant polysaccharide and an important component in plant cell wall. The plant cell wall is made of composite materials in which cellulose, hemicelluloses (mainly xylan) and lignin maintains the plant cell wall structural integrity through covalent linkage bond of xylan with lignin and non-covalent linkage bond of xylan with cellulose. Xylan is present in cell wall material of all terrestrial plant, annual plants (30%), hard woods (15-30%) and soft woods (7-10%) (Subramaniyan & Prema, 2002). Xylan is a complex branched heteropolysaccharide. The β-1,4-linked xylopyranosyl residues which made xylan main chain have a side group substitution to varying degree with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, β-L-arabinofuranosyl, acetyl, feruloyl and p-coumaroyl groups (Kulkarni *et al.*, 1999; Li *et al.*, 2000, as cited in Kuhad & Singh, 2007).

Due to heterogeneity and complexity of xylan structure, its degradation required a variety of hydrolatic enzymes working together (Beg *et al.*, 2001). For this purpose, the xylan degrading cells produce a set of enzyme with difference specificity and modes of action for complete hydrolysis of xylan. Xylan back bones at endo-β-1,4-xylosidic linkage is randomly cleave by endo-1,4-β-D-xylanase (EC 3.2.1.8), releasing xylooligosaccharides while β-D-xyloidases (EC 3.2.1.37) cleave the β-1,4-xylosidix linkage terminal, releasing monomers from the reducing end of xylooligosaccharides and xylobiose. The removal of side group is carried out by α-L-atabinofuranosidases (EC 3.2.1.55), α-L-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.2.1.72) and ferulic acid esterases (EC

3.1.1.73). All this mentioned enzyme made up the xylanolytic enzyme system (Kuhad & Singh, 2007).

2.2 Xylanase

In this study, the focus was on endo-1,4-β-D-xylanase (EC 3.2.1.8). Xylanase cleave the xylan backbone at 1-4 carbon linkage to produce xylooligosaccharides and xylose (Kulkarni *et al.*, 1999). Various organisms have been reported to be able to produce xylanase such as bacteria, fungi, yeast,, protozoans, marine algae, snails, crustaceans, insects and seeds (Kuhad & Singh, 2007). However, there are also studies showing some higher animal such as freshwater mollusk capable of producing xylanase. Furthermore, there are also cases of plants such as Japanese pear which produce xylanase during the over ripening period in a fruit cycle (Subramaniyan & Prema, 2002).

Xylanase exist in numerous forms, displaying varying folds, mechanisms of action, substrate specificities, hydrolytic activities and physicochemical characteristics. These enzymes would later be grouped according to primary structure comparisons of catalytic domain and sequence similarity into families of glycosyl hydrolase (Collins *et al.*, 2005). Xylanase are mostly classified based on sequence similarities into two family of glycosyl hydrolase, which is family 10 and family 11 (Henrissat, 1991). Usually, xylanase that belong to family 10 have larger molecular mass (more than 40 kDa) when compared to those belong to family 11 (around 20 kDa). For application of xylanase in industry such as biobleaching, the xylanase from family 11 are more efficient when compared to that of family 10 xylanase since it have smaller molecular mass, more compact and no cellulase

activity, making it easy to penetrate the cellulose fiber network without destroying the fiber (Torronen & Rouvinen, 1997; Oakley *et al.*, 2003, as cited in Helianti *et al.*, 2008).

Apart from that, xylanase can be classified as either specific or non-specific based on their action on different polysaccharides (Coughlan, 1992; Coughlan *et al.*, 1993, as cited in Bedford & Partridge, 2001). The specific xylanase are active on xylan with only β-1,4 linkages. Non-specific xylanase hydrolyze β-1,4 linked xylans, β-1,4 linkages of mixed xylan and other β-1,4 linked polymers such as CMC-cellulose. As the matter of fact, the determination of the kinetic constants ratio k_{cat}/k_m for xylan and CM-cellulose for a particular non-specific xylanase should confirm whether the enzyme in question is either a xylanase or cellulose (Bedford & Partridge, 2001).

Xylanase attracted research interest because of its high potential in industrial applications. From biotechnology perspective, xylanase is an important enzyme since it can work alone or with the combination with others enzyme. In addition, xylanase thermal stability makes it suitable in a number of industrial applications (Te'o *et al.*, 2000). One of many useful applications of xylanase is in paper and pulp industry where it is use in kraft process and biobleaching. Xylanase is also a promising enzyme in the development of environmentally friendly in paper and pulp industry through the development of cellulase-free xylanase. Other than that, xylanase is use in bakeries where it helps to improve the bread desirable texture, loaf and shelf life. Xylanase also works with other enzymes to convert xylan into xylose, xylitol, art paper and bioethanol (Kulkarni *et al.*, 1999; Helianti *et al.*, 2008).

2.3 Xylanase from *Klebsiella pneumoniae*

In this study, a heterologous expression of gene encoding β-1,4-endoxylanase in *E. coli* BL21 (DE3) was performed. The xylanase gene was isolated from xylanolytic *Klebsiella pneumoniae (K. pneumoniae),* a bacterium that had been locally isolated from soil at the vicinity of the sago plantation by Hussain *et al.* (2011). The isolated bacteria were identified as gram negative rods with milky, circular, convex colonies. The bacteria are non-motile and tested positive for both Voges-Proskauer test and citrate utilization test, and negative for H_2S production (Hussain *et al.*, 2011).

The isolated xylanase gene is from family 11 glycoside hydrolase and showed similarity to *Bacillus* sp., although 16 rRNA showed that the bacterium closely resembled *Klebsiella*. *In-silico* characterization of the isolated xylanase gene showed that it has an ORF of 642 bp encoding 213 amino acids, and are predicted to code for xylanase with a molecular weight 23.3 kDa with an isoelectric point of 9.44, which is quite similar to the xylanase isolated by Jalal *et al.* (2009) from *B. subtilis*, as well as Helianti *et al.* (2008) and Lee *et al.* (2008) from *B. licheniformis*. The isolated xylanase showed 99.5% identity to the xylanase isolated by Jalal *et al.* (2009). However, only 91% identity was found when compared to the xylanase isolated by Helianti *et al.* (2008) and Lee *et al.* (2008). The low identity when compared with *B. licheniformis* isolated by Helianti *et al.* (2008) and Lee *et al.* (2008) is due to the thermophilic adaptation of *B. licheniformis* (Hussain *et al.*, 2011).

So far, there are no research publications that described the isolation of xylanase gene from *K. pneumoniae*. Xylanase gene is commonly isolated from genus *Bacillus*, thus the isolation of xylanase gene from *K. pneumoniae* suggests that xylanase might be naturally occurring among soil-inhabiting microbes. The isolated xylanase showed a high conservation, implying that these xylanase play an important role in the natural

environment for the degradation of xylan (Hussain *et al.*, 2011). This forms of xylanase have no cellulose activity (Lee *et al.*, 2008), thus it can be potentially used in applications such as biobleaching of paper pulp that requires the removal of hemicelluloses but with intact cellulose (Hussain *et al.*, 2011).

2.4 Recombinant Xylanase

A heterologous expression of gene encoding β-1,4-endoxylanase in *E. coli* BL21 (DE3) was performed in this study. Heterologous expression of a gene refers to the fact that the transferred gene was initially cloned from a different species than the recipient. In actuality, the gene itself is not transferred to the recipient cell, but rather the 'correctly edited' DNA coding for the protein. The DNA that is transferred must be within a format that encourages the recipient to express DNA as a protein, and for that matter the recipient that is use must be an expression vector (Mus-Veteau, 2002). Currently, the heterologous expression has become one of the main tools for the production of industrial enzymes (Kirk *et al.*, 2002, as cited in He *et al.*, 2009). *E. coli* is the most commonly used host for the metabolic engineering and recombinant protein production (Ni & Chen, 2009). *E. coli* is commonly selected than fungal since its productivity is higher due to its fast growth (Farliahati *et al.*, 2009).

Recombinant xylanase characteristics have been analyzed and compared to its native xylanase in quite a large number of research publications under the varying degree of temperature and pH. The crude xylanase enzyme activity for the isolated *K. pneumoniae* is relatively low, but the recombinant xylanase in this study is expected to show a high increase in activity once expressed and purified. The assumption is based on

research done by Jalal *et al.* (2009), Helianti *et al.* (2008), and Lee *et al.* (2008), since their recombinant xylanase showed high increase in activity compared to the activity by native xylanase. Cloning of xylanase gene into an *E. coli* expression host had been shown to successfully increase the activity of recombinant xylanase in many research publications (Yang *et al.*, 1988; Whitehead & Hespell, 1989; Helianti *et al.*, 2008; Lee *et al.*, 2008; Jalal *et al.*, 2011). Apart from that, the recombinant xylanase might have slightly different properties in term of pH and temperature stability when compared to that recombinant xylanase by Jalal *et al.* (2009), Helianti *et al.* (2008), and Lee *et al.* (2008). This is due to the slight difference in amino acids sequence.

Recombinant xylanase had been reported to be distributed in extracellular, intracellular and periplasmic fractions in genus *Bacillus* (Huang *et al.*, 2006). Apart from that, recombinant xylanase were commonly found to be in insoluble fraction of cytoplasmic, hinting that even after optimization the expression of xylanase gene produces protein that is insoluble and accumulates in inclusion bodies (Yin *et al.,* 2008). Secretion of non-glycosylated recombinant xylanase had also been observed (Kulkarni *et al.*, 1999). Generally, the production of xylanase through recombinant technology is of economical benefits aiming toward higher productivity and trouble-free downstream processing in fermentation (Farliahati *et al.*, 2009). Besides that, recombinant xylanase enzymes can be used for cellulose-free xylanase production in case of maintaining the cellulose structure as well as providing tolerances to various cations (Lee *et al.*, 2008).

3.0 MATERIALS AND METHODS

3.1 Xylanase Template Preparations

3.1.1 Culturing *E. coli* JM109

Agar plate containing *E. coli* JM109 single colonies was obtained from UNIMAS Proteomic Laboratory. By using aseptic technique, a single colony from the *E. coli* JM109 single colonies was taken using inoculating loop and then streaked into a new LB agar plate. Then, the streaked agar plate was placed inside an incubator and leaved overnight at 37°C. After overnight incubation, the streaked agar plate which now has *E. coli* JM109 single colonies growth in it was stored at 4°C until further use.

3.1.2 Overnight Bacterial Cells Culture Preparations

A single colony was inoculated aseptically from the agar plates containing *E. coli* JM109 single colonies using inoculating loop. Then, the *E. coli* JM109 single colony was transferred aseptically into a sterile bijou bottle containing 5 mL sterile LB media. The culture then was incubated with shaking for overnight at 37°C. The bacterial cultures were stored at 4°C until further use.

3.1.3 Calcium Chloride ($CaCl_2$) Bacterial Competent Cells Preparation

Approximately 5 mL of the overnight bacterial cells culture was added to an Erlenmeyer flask containing 50 mL of pre-warmed LB media without any antibiotics, and allowed to grow at 37°C with shaking at 250 rpm until the OD_{600} reaches approximately 0.45-0.5. The process takes about 1 hour and 20 minutes. The flask was cooled on ice for 10 to 20 minutes. The cells suspension was centrifuged at 3500 rpm at 4°C for 5 minutes in

cooled McCartney bottles or polypropylene centrifuge tubes. The supernatant was discarded and the cells were washed gently by resuspending them in 25 mL iced-cold 100 mM $CaCl_2$. The cells suspension was kept on ice for 10 minutes, and then was centrifuged again as mention above. After centrifugation, the supernatant was decant and the cell pellet was resuspended in 2.5 mL of cold sterile 100 mM $CaCl_2$. At this point, glycerol stock, which can be stored for long period of time at 80°C, was prepared by the addition 0f 20% (v/v) pure glycerol to the cells suspension. The mixture was mixed well, and 200 µL aliquots were transferred into 15 Eppy tubes, followed by storage at -80°C (ultra-freezing).

3.1.4 Heat Shock Transformation into *E. coli* JM109 Protocols

Recombinant pGEM-T Easy Vector that has xylanase gene insert and recombinant pSTAG expression vector that has *stisa* gene insert was obtained from the UNIMAS Proteomic Laboratory. Firstly, 1 µL of recombinant pGEM-T vector and 1 µL of recombinant pSTAG vector were added into two Eppendorf tube respectively, where each of the tube contain 25 µL of competent cell, *E. coli* JM109. The mixture was mixed by stirring using a pipette tip. The cells were then incubated on ice for 20 min and then heat shocked for 45s at 42°C. Then the cells were kept in ice for 2 minutes. After that, 1 mL of LB was added into the tube and the tube then was incubated at 37°C with shaking at 150 rpm for 2 hours. The tube was centrifuged at 14,000 rpm for 5 min. Approximately 800 µl supernatant was discarded and the pellet was dissolved in remaining broth. Transformation culture was spread onto LB plates that contain ampicillin (50 µg/mL). The culture was incubated at 37°C for -18 hours.

3.1.5 Plasmid DNA Extraction from Bacterial Cultures Protocol

Overnight bacterial cells culture was prepared using the *E. coli* JM109 of recombinant pGEM-T vector and recombinant pSTAG vector, which was incubated with shaking at 37°C for overnight in LB media containing ampicillin (50 µg/mL). Approximately 1.5 mL overnight culture was harvested by transferring it into a microcentrifuge tube and then centrifuging at 14,000 rpm for 2 minutes at room temperature. The supernatant (culture media) was removed carefully, and the pellet was re-centrifuged for 1 minute. Any trace of liquid media was removed completely from the tube.

The cell pellet was resuspended by vortexing briefly for 10 seconds using 100 µL of Resuspension Solution (50 mM Glucose, 1.8 M Formic Acid and 25 mM Tris-HCl, pH 8). The tube then was kept on ice after that. Then, 100 µL of Lysis Solution (0.2 N NaOH and 1% SDS) was added to the cell suspension and then was mixed gently by inverting the tube 10x. The tube was left at room temperature to allow lysis reaction to occur for 5 minutes, and was made sure not to exceed 5 minute. After that, a clear viscous liquid are observed. Then, 300 µL of Neutralization Solution (3 M Potassium Acetate and 10 mM EDTA, pH 8) was added and then mixed by inverting the tube 10x. A white precipitate was observed. The precipitate was pelleted by centrifuging at 14,000 rpm for 5 minutes. The supernatant (containing plasmid DNA) was carefully transferred into a sterile 1.5 mL Eppy tube.

The DNA was precipitated by adding 2 volume of cold absolute ethanol. The content was mixed gently by inverting the tube at least 10x. The DNA was pelleted by centrifuging at 14,000 rpm for 5 minutes at room temperatures. The supernatant was discarded and the pellet was washed with 500 µL of 70% ethanol, and recentrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded as much as possible and the